Different Cell Compositions in Mungbean (*Vigna radiata* (L.) Wilczek) Cotyledon Calli

Josefina O. Narciso and Kazumi Hattori

Laboratory of Plant Breeding and Genetics, School of Agricultural Sciences, Nagoya University, Chikusa, Nagoya 464-01, Japan

Summary

Two types of cell masses namely, green and compact (GC) and white and friable (WF) were initiated from a single, identical callus mass of the mature cotyledon of mungbean strain IPB 22-117 upon subculture. Cryo-SEM examination of these two types of cell masses revealed distinct cell compositions. The GC mass was composed mainly of small, round, closely-packed cells while the WF mass was of the elongated, loosely-packed cells. Occasionally, a few big round cells were found among elongated cells. Differential response of the two types of masses in suspension culture was also observed. Of the two, the WF mass gave a fine cell suspension in L6 medium (Kumar *et al.*, 1988). Light microscopic observations of the WF cell suspension under varying periods of culture showed different cell types. When plated, big, round cells generally undergo cell division. Colony formation was not profuse among elongated cells and most of them remained in their original state.

Key Words : cell types, small round cells, big round cells, elongated cells, mungbean calli, cell suspension.

Introduction

Varietal improvement in mungbean (*Vigna radiata* (L.) Wilczek) has been mainly through conventional breeding method. Despite many improvements brought about by this strategy, the development of superior genotypes using this method is rather slow. A better approach in facilitating plant improvement in this crop is to employ non-conventional strategy such as cell and tissue culture along with the conventional ones. In particular, manipulations at the cellular level will bring great impact in its plant breeding program.

Fundamental to the exploitation of tissue culture is the requirement to regenerate plants routinely and in sufficient numbers from cultured cells (Hammat *et al.*, 1986). Although *in vitro* studies on this crop are expanding, protocol for regeneration from established callus and/or cultured cells has not been fully established. Until today, mungbean is considered recalcitrant. What makes mungbean cells incapable of regenerating remains a challenge to researchers.

There has been no known reports about the cell composition in a callus mass and its possible relationship to

regeneration. Most of the past works dealt on somatic embryogenesis and described the characteristics of the cell present in an embryogenic culture. In mungbean and related species, Eapen and George (1990) reported that cells destined to become somatic embryos were generally round or oblong in shape. These cells divided to form spherical or filamentous proembryo. They did not however, study the cell compositions of the callus mass that they used to initiate the cell suspension.

Callus is usually the starting material for single cell selection and regeneration studies via somatic embryogenesis. And it is very essential to know whether a callus mass is made up of embryogenic or non-embryogenic cells. In mungbean, the cell composition of a single callus has not been studied yet. Along this line, this study was conducted to examine the different cell compositions in mungbean cotyledon calli and determine their response under liquid and solid media.

Materials and Methods

Callus Induction

Seeds of mungbean strain IPB 22-117 were used as the test material for this study. The seeds were kindly provided by the Institute of Plant Breeding of the University of the Philippines at Los Banos, College, Laguna. The seeds were surface sterilized following the method of Mendoza and Futsuhara (1990) and imbibed in sterile distilled water for 16 hrs. After careful removal of the seedcoat, the cotyledons were excised and the embryonic axes were completely removed. The cotyledons were cultured with their rounded side in contact with the medium. The callus induction medium by Eapen and George (1990) composed of MS salts (Murashige and Skoog, 1962), B5 vitamins (Gamborg *et al.*, 1968), 5 mg/l 2,4-dichlorophenoxyacetic acid (2,4-D), 3 % sucrose and 0.7 % agar was used to induce callus from the cotyledons. The initiated calli were first subcultured in the same medium but with the addition of 3 mg/l 2,4-D and 1 g/l casein hydrolysate hereafter referred to as S1. For the second subculture, the same S1 components were used but with reduced level of 2,4-D at 2 mg/l, hereafter referred to as S 2.

Cell Suspension

The calli proliferated from the second subculture were used to initiate the cell suspension. The calli were cultured in the callus induction medium by Eapen and George (1990) but without the agar component. About

200 mg of calli were placed in 100 ml Erlenmeyer flask containing 40 ml of the liquid medium. The cultures were incubated on a reciprocating shaker at 120 rpm under continuous light (5,000 lx) at $25 \pm 2^\circ\text{C}$. Subculture of the suspension culture was done at two-week interval. In the first and second subcultures, S 1 and L 6 (Kumar *et al.*, 1988) were the culture media used. The cell suspension was transferred into 50 ml tube and centrifuged at 1,000 rpm for 10 min. The old medium was pipetted out and the cells were resuspended in 40 ml of the fresh medium. Twenty ml of the resuspended cell were transferred to another flask containing equal volume of the fresh medium. At the third subculture, the cells were cultured in L 6 with 0.08 mg/l kinetin (Ki) and PC-L 2 basal medium developed by Phillips and Collins (1979) supplemented with 1 mg/l naphthaleneacetic acid (NAA) and 0.1 mg/l 6-benzyladenine (6-BA).

Plating

Three weeks after the third subculture, the cells were plated in the following media: a) MS salts supplemented with B5 vitamins, 5 mg/l 2,4-D and 30 g/l sucrose b) B5 salts and vitamins with 1 mg/l 2,4-D, 1 mg/l Ki and 20 g/l sucrose, c) L6 salts and vitamins (Kumar *et al.*, 1988) with 0.5 mg/l 6-BA and 20 g/l sucrose. Four ml of the cell suspension were transferred into 10 ml test tube and centrifuged at 1,000 rpm for 5 min. The old medium was pipetted out and replaced with 10 ml of the plating liquid medium. The cells were resuspended and placed into 90 x 20 mm disposable petri dish. An equal amount of plating medium with agar (approximately 40°C) was mixed with the cell suspension. Each dish was sealed with parafilm and incubated at $25 \pm 2^\circ\text{C}$ under continuous light (5,000 lux).

Calli induced during plating were transferred to four different regeneration media namely: 1) L 6 salts + B5 vitamins + 100 mg/l myo-inositol + 200 mg/l casein hydrolysate + 10 g/l sorbitol + 2.25 mg/l 6-BA + 20 g/l sucrose 2) PC-L 2 salts and vitamins + 0.06 mg/l picloram + 1.0 mg/l 6-BA + 25 g/l sucrose 3) MS salts and vitamins + 0.2 mg/l NAA + 2 mg/l 6-BA + 100 mg/l yeast extract + 30 g/l sucrose 4) B 5 salts and vitamins + 0.2 mg/l NAA + 2 mg/l 6-BA + 30 g/l sucrose.

The pH of all media was adjusted to 5.8 prior to addition of agar and autoclaved at 121°C for 20 min at 1.2 kg/cm^2 .

For scanning electron microscopic observation, samples were dipped in liquid nitrogen and viewed under an Hitachi S-2300 scanning electron microscope with cryo-stage.

Results

Callus Initiation

The medium as described by Eapen and George (1990) proved to be appropriate for callus induction of IPB 22-117. A week after culture, callus formation was

observed starting at the periphery of the cotyledon and gradually covering the whole explant. The explant was fully covered with the green compact callus mass within six weeks of culture. Three weeks after subculture, callus mass exhibited morphological changes. Color pigmentation as well as change in texture were very evident. The portion in contact with the medium became white and friable (WF) while the top portion remained green and compact (GC) (Fig 1). Everytime the GC portion was subcultured, the same morphological changes were observed: the portion in contact with the medium became WF and the top remained GC. This observation was consistent with the results of the preliminary study of the authors.

Cell compositions of the subcultured callus mass

Samples for scanning electron microscopy were taken from three portions of a callus mass: the bottom portion which was directly in contact with the medium, the middle portion and the top portion which was not directly in contact with the medium.

Scanning electron microscopic observation of each portion, revealed distinct cell compositions (Fig 2 a). The bottom portion which was WF was composed mainly of elongated cells which were loosely-packed (Fig 2 b). In the middle portion, mainly WF, and with some GC mass, few big, round cells (BR) were found among groups of elongated cells (ELC) (Fig 2 c). In contrast, the top portion which was GC was made up of small, round, closely-packed cells (SR) (Fig 3 a). Thus in general, there were three cell types: BR, SR and ELC. Visible sign of cell division was frequently seen among the BR (Fig 3 b). Such was not observed in ELC during the time of observation.

Response of the different cell masses in cell suspension cultures

For cell suspension induction, the callus mass was

Fig. 1. Callus of IPB 22-117 during subculture showing the different callus masses. (Abbreviations: gc=green, compact; wf=white, friable).

Fig. 2 a-c. Scanning electron microscopic observations of the different layers of the callus mass shown in Fig. 1. (a) Elongated cells (ec) in the layer in contact with the medium and round cells (rc) on the top layer (b) Elongated, loosely-arranged cells of the white, friable callus type at the bottom layer. (c) Predominantly elongated cells with few big, round cells (arrow) at the middle layer. Scale bars = 100 μm .

divided into two portions, top and bottom. The top portion was composed mainly of the GC mass while the bottom portion was of the WF mass.

A total of five WF lines and six GC lines were evaluated. Each line was cultured in S 1 and L 6 with 24 flasks per medium. Cell suspension cultures of the two types of cell masses showed marked differences. Because of its friable nature, all the WF lines developed finely dispersed suspension in both culture media tested. The GC lines failed to develop good suspension but rather callus aggregates which turned brown upon prolonged culture (Fig 4 a). Of the five WF lines, two lines were maintained because of their good quality suspension characterized by finely dispersed greenish color suspension (Figs. 4 b and 4 c). Between S 1 and L 6, L 6 was the much better medium for cell suspension culture. L 6 produced good quality suspension with very minimal browning. At the third subculture, good quality suspension was maintained in L 6 supplemented with kinetin while those transferred to PCL 2 developed into

Fig. 3 a and b. Scanning electron microscopic observations of the green, compact callus mass showing two types of round cells. (a) Small, round, closely-packed cells of the green, compact callus mass at the top layer (b) Early signs of cell divisions in big, round cells (arrow). Scale bars=100 μm .

brownish aggregates.

Cell types in suspension culture

Different cell types were noted in the WF suspension. Elongated and round cells were observed. The round cells might have come from the GC cell mass which was accidentally taken during sampling. Among the elongated cells, gourd-shaped and bean-shaped cells were observed (Fig. 5 a and 5 b). They were in general, highly vacuolated and appeared as single cells. On the other hand, the round cells were either perfectly round or egg-shaped with rich cytoplasm. They were also clustered (Fig 5 c). Toad pole-shaped cells were also noted (Fig. 5 b). These might have arisen as a result of transformation in elongated and round cells.

Cell division and colony formation in plated suspension cell

In the plated suspension cell, profuse cell division was observed 11 days after initiation of culture. In general, BR cells undergo cell division as shown by the formation of cell clusters (Figs. 6 a and 6 b). On the other hand, ELC very rarely undergo cell division with most of them remaining in their original state (Fig 6 c). Among the three plating media, colony formation was fastest in B 5 followed by MS. Colony formation was

Fig. 4. Response of the different callus types in cell suspension cultures. (a) Brownish, callus aggregates of the green, compact callus type in L 6 medium. (b and c) Finely dispersed suspension of the white, friable callus line in L 6 and MS media, respectively.

Fig. 5 a-c. Light microscopic observations of the 3-week old white, friable cell suspension. (a) gourd-shaped elongated cell (arrow) (b) bean-shaped elongated cells (arrow) and tadpole-shaped cells (double arrows) (c) clustered, big, round cells. Scale bars = 100 μ m.

very late in L 6. Colonies in B 5 appeared as brownish dots while those in L 6 and MS were whitish.

Fig. 6a-c. Light microscopic observations of the plated suspension cell. (a) Cluster of big, round cells in MS medium. (b) Cluster of big, round cells in B 5 medium. (c) Single cells of the elongated type in B 5 medium. Scale bars = 100 μ m

Response to regeneration

Twenty six days after plating, calli of friable, brownish green type have started emerging on top of the B 5 medium. Callus formation in MS was observed 2 weeks later. Calli induced in MS were friable and greenish in color. When calli reached the size of about 2 mm in diameter, they were cultured in the regeneration media as described in the materials and methods. The only visual response observed in these cell suspension-derived calli were callus expansion and greening noted in PC-L 2 and L 6. Progressive browning of calli was observed in MS and B 5-based media. Regeneration was not attained in all media tested even after more than 60 days of culture.

Discussion

Cell types appear to strongly govern cell mass types and their response in liquid medium. Cell division is also seemed to be influenced by cell types. The WF cell mass consisting of elongated, loosely-packed cells was more amenable to agitation and therefore produced good suspension. In contrast, the GC cell mass consisting of small, round, closely-packed cells was very hard to in-

duce good suspension under any medium tested. Elongated cells very rarely undergo cell division, highly vacuolated and appeared mostly as single cells. Such are the known features of non-embryogenic cells as noted by previous workers (Lu and Vasil, 1981 ; Ho and Vasil, 1983; Huang *et al.*, 1993). The round cells usually undergo profuse cell division and were clustered which are the general characteristics of embryogenic cells. Previous works by Ho and Vasil (1983) in sugar cane, Huang *et al.*, (1993) in barley, Vasil and Vasil (1981) in pearl millet and by Lu and Vasil (1981) in guinea grass reported similar features of embryogenic cells. In this study, results suggest that the round, closely-packed cells in GC cell mass type were embryogenic while the elongated, loosely-packed cells of the WF cell mass type were non-embryogenic.

Differential response to media compositions was observed during cell suspension culture. L 6 was noted to be better than S 1 in maintaining good quality suspension. Similar findings were noted by Kumar *et al.*, (1988) in the cell suspension of *Vigna aconitifolia*. L 6 medium differs from other known media such as MS and B 5 in having a lower level of inorganic nitrogen and increased calcium. The good response of culture in L 6 can be attributed to these deviations along with the addition of B 5 vitamins and casein hydrolysate.

Regeneration from cell-suspension-derived calli was not attained in this study. Introduction of other components in the medium might modify callus texture and induce embryogenesis such as proline (Armstrong and Green, 1985), high levels of benzylamino-purine (Lazzeri *et al.*, 1987), amino acids as sources of exogenous nitrogen (Toriyama *et al.*, 1986), and silver nitrate (Vain *et al.*, 1989). In legumes, growth regulators and reduced nitrogen sources exert significant effect on both embryogenesis and caulogenesis (Hammat *et al.*, 1986). Moreover, other factors such as light and temperature must be considered.

Results of this study revealed that in mungbean from a single, identical callus mass, two cell mass types namely GC and WF were initiated during subculture. The two cell mass types were produced each time the top, GC cell mass was subcultured. Each cell mass type showed distinct cell types.

Literature Cited

- Armstrong, C. L. and C. E. Green (1985) Establishment and maintenance of friable, embryonic maize callus and the involvement of L-proline. *Planta*. 164 : 207-214.
- Chee, R. P. and D. J. Cantliffe (1989) Composition of embryonic suspension cultures of *Ipomea batatas* Poir. and production of individualized embryos. *Plant Cell, Tiss. Org. Cult.* 17 : 39-52.
- Eapen, S. and L. George (1990) Ontogeny of somatic embryos of *Vigna aconitifolia*, *Vigna mungo* and *Vigna radiata*. *Ann. Bot.* 66 : 219-226.
- Gamborg, O. L., R. A. Miller and K. Ojima (1968) Nutrient requirements of suspension cultures of soybean root cells. *Exp. Cell Res.* 50 : 151-158.
- Hammat, N., T. K. Ghose and M. R. Davey (1986) Regeneration in legumes. In : Vasil IK (Ed) *Cell Culture and Somatic Cell Genetics of Plants*, Vol. 3 (pp 67-95). Academic Press, Orlando.
- Ho, W.-J. and I. K. Vasil (1983) Somatic embryogenesis in sugar-cane (*Saccharum officinarum* L.) : Growth and plant regeneration from embryogenic cell suspension cultures. *Ann. Bot.* 51 : 719-726.
- Huang, C., H. Yan, Q. Yan, M. Zhu, M. Yuan and A. Xu (1993) Establishment and characterization of embryogenic cell suspension cultures from immature and mature embryos of barley (*Hordeum vulgare* L.). *Plant Cell, Tiss. Org. Cult.* 32 : 19-25.
- Jones, L. H. (1974) Factors influencing embryogenesis in carrot cultures (*Daucus carota* L.). *Ann. Bot.* 38 : 1077-1088.
- Kott, L. S. and K. J. Kasha (1983) Initiation and morphological development of somatic embryos from barley cell cultures. *Can. J. Bot.* 62 : 1245-1249.
- Kumar, A. S., O. L. Gamborg and M. W. Nabors (1988) Plant regeneration from cell suspension cultures of *Vigna aconitifolia*. *Plant Cell Rep.* 7 : 138-141.
- Lazzeri, P. A., D. F. Hilderbrand and G. B. Collins (1987) Soybean somatic embryogenesis : Effects of hormones and culture manipulations. *Plant Cell, Tiss. Org. Cult.* 10 : 197-208.
- Lu, C.-Y. and I. K. Vasil (1981) Somatic embryogenesis and plant regeneration from freely-suspended cells and cell groups of *Panicum maximum* Jacq. *Ann. Bot.* 48 : 543-548.
- Mendoza, A. B. and Y. Futsuhara (1990) Varietal differences on plant regeneration by tissue culture in mungbean *Vigna radiata* (L.) Wilczek. *Japan J. Breed.* 40 : 457-467.
- Murashige, T. and F. Skoog (1962) A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol. Plant.* 15 : 473-497.
- Phillips, G. C. and G. B. Collins (1979) In-vitro tissue culture of selected legumes and plant regeneration from callus cultures of red clover. *Crop Sci.* 19 : 59-64.
- Toriyama, K., K. Hinata and T. Sasaki (1986) Haploid and diploid regeneration from protoplasts of anther callus in rice. *Theor. Appl. Genet.* 73 : 16-19.
- Vain, P. H. Yean and P. Flament (1989) Enhancement of production and regeneration of embryogenic type II callus in *Zea mays* L. by AgNO₃. *Plant Cell Tiss. Org. Cult.* 18 : 143-151.
- Vasil, V. and I. K. Vasil (1981) Somatic embryogenesis and plant regeneration from suspension cultures of pearl millet (*Pennisetum americanum*). *Ann. Bot.* 47 : 669-678.